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PHENYLIMINOIMIDAZOLIDINE DERIVATIVES ACTIVATE BOTH OCTOPAMINE$_1$ AND OCTOPAMINE$_2$ RECEPTOR SUBTYPES IN LOCUST SKELETAL MUSCLE

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SUMMARY

1. The actions of the phenyliminoimidazolidine derivatives, NC7 and NC5 on octopamine receptors in the extensor-tibiae neuromuscular preparation of the locust are described.

2. The derivatives activate both OCTOPAMINE$_1$ and OCTOPAMINE$_2$ receptors in this preparation. They are not selective agonists of OCTOPAMINE$_2$ receptors that activate adenylate cyclase.

3. The relative potencies of NC5 and NC7 on OCTOPAMINE$_1$ and OCTOPAMINE$_2$ receptors differ, suggesting that they may be useful in the development of selective agonists that can distinguish between the different pharmacological subclasses of octopamine receptor.

4. The octopamine-mediated increases in cyclic AMP levels in this preparation can be separated into high- and low-affinity components. NC5 and NC7 appear to activate the high-affinity component of this response selectively.

INTRODUCTION

A considerable amount of evidence suggests that the biogenic amine octopamine functions as a neuromodulator, a true neurotransmitter and a circulating neurohormone in insects (see Evans, 1985*a*). Multiple pharmacologically distinct classes of octopamine receptor with different modes of action have been demonstrated in an insect preparation (Evans, 1981, 1984*a*,*b*,*c*) which parallels similar studies on biogenic amine receptors in other preparations (see Snyder & Goodman, 1980; Berridge & Heslop, 1981). However, the bulk of useful information about the functional roles of octopamine receptors in insects has been obtained from peripheral systems such as firefly light organs (Nathanson, 1979; Christensen & Carlson, 1981, 1982), locust corpora cardiaca (Orchard, Gole & Downer, 1983), insect visceral muscle (Lange & Orchard, 1984; Orchard & Lange, 1985, 1986) and insect skeletal muscle (Evans & O'Shea, 1977, 1978; O'Shea & Evans, 1979; Evans & Siegler,

Key words: phenyliminoimidazolidines, octopamine receptors, insect muscle.

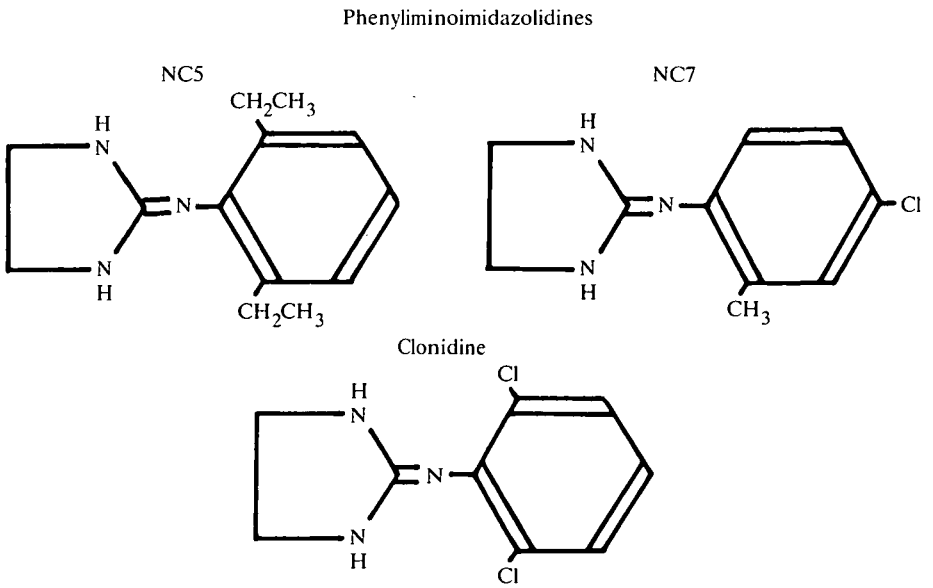

Fig. 1. Structures of the phenyliminoimidazolidine derivatives NC5, NC7 and clonidine.

1982). If meaningful studies of octopamine receptor function are to be extended to the insect central nervous system, then selective ligands are required which will distinguish octopamine receptors from the receptors present for other biogenic amines and, in addition, differentiate between the various pharmacological subclasses of octopamine receptor.

It has been suggested that various phenyliminoimidazolidine derivatives may be agonists for $OCTOPAMINE_2$ receptors that mediate their actions *via* increasing cyclic AMP levels (Nathanson, 1985*a*) and that, further, the relative potencies of some of these derivatives could distinguish between various subtypes of $OCTOPAMINE_2$ receptors present in different insect preparations (Nathanson, 1985*a*,*b*). However, these claims were made on the basis of results obtained in preparations in which only $OCTOPAMINE_2$-receptor-mediated physiological and biochemical effects were studied. In addition, the claims for the distinction of $OCTOPAMINE_2$ receptor subtypes were based on measurements of total agonist-induced changes in cyclic AMP levels without knowledge of how much of the effect was actually mediated *via* an activation of specific octopamine receptors.

The present paper reports on the actions of the phenyliminoimidazolidine derivatives, NC5 and NC7, on octopamine receptors in the extensor-tibiae muscle preparation of the locust. Fig. 1 shows the structure of these derivatives compared to clonidine, an agonist previously reported to be active on octopamine receptors in this preparation (Evans, 1981). This preparation was chosen for study since it contains both $OCTOPAMINE_2$ receptors, that mediate their effects by increases in cyclic AMP levels (Evans, 1984*a*,*b*), and $OCTOPAMINE_1$ receptors, that do not appear

to mediate their effects *via* increases in cyclic AMP levels but which may act by releasing calcium from intracellular stores (Evans, 1984*c*).

MATERIALS AND METHODS

Adult locusts (*Schistocerca gregaria*) of either sex were obtained from crowded laboratory colonies fed on wheat seedlings. Small batches of animals were left for 1–2 h before use, after removal from the main culture, to minimize any initial potentiation effects due to elevated levels of octopamine in the haemolymph (see Evans, 1981; Davenport & Evans, 1984*a*,*b*).

Tension in the extensor-tibiae muscle of the locust hindleg was measured almost isometrically with a tension transducer attached distally to the apodeme. The slow extensor-tibiae (SETi) motor neurone was excited by stimulating nerve 3b with a pair of silver hook electrodes. An operational amplifier signal differentiator was used to measure continuously the rates of neurally evoked contraction and relaxation (Buchan & Evans, .1980).

Extensor-tibiae muscles were prepared for cyclic AMP measurements by incubation as described previously (Evans, 1984*a*). At the end of the incubation period, muscles were rapidly frozen and dissected away from their surrounding cuticle. The muscles were homogenized in an ice-cold concentrated hydrochloric acid:absolute ethanol mixture (1:60 v/v) (Horn & McAfee, 1977). The cyclic AMP levels were assayed in the muscle extracts by a protein-binding method (Brown, Ekins & Albano, 1972) using a commercial cyclic AMP assay kit (Amersham). Protein determinations were carried out according to the method of Lowry, Roseburgh, Farr & Randall (1951) using bovine serum albumin as standard.

Drugs were superfused directly onto the surface of the muscle. They were dissolved in a physiological isotonic saline (pH 6·8) containing (in mmol l^{-1}) NaCl, 140; KCl, 10; $CaCl_2$, 4; $NaHCO_3$, 4; NaH_2PO_4, 6; sucrose, 90. Phentolamine mesylate was a gift from CIBA and (−)-para-octopamine was kindly supplied by Professor J. M. Midgley, Department of Pharmacy, University of Strathclyde, Glasgow. Samples of NC5 and NC7 were kindly supplied by Dr J. A. Nathanson, Department of Neurology, Massachusetts General Hospital, Boston.

RESULTS

Effects on OCTOPAMINE$_2$ receptors modulating neuromuscular transmission

Neuromuscular transmission mediated by the slow excitatory motor neurone (SETi) to the extensor-tibiae muscle of the locust metathoracic leg is modulated by both presynaptic and postsynaptic octopamine receptors which have been designated OCTOPAMINE$_{2A}$ and OCTOPAMINE$_{2B}$ receptors, respectively (Evans, 1981). The phenyliminoimidazolidine derivatives, NC5 and NC7, are potent agonists of both of these subtypes of octopamine receptor. Fig. 2 shows that both NC5 and NC7 can increase the amplitude of SETi-induced twitch tension in a dose-dependent manner. At low concentrations in the range 10^{-9}–10^{-7} mol l^{-1} the effects of NC5 and

NC7 are not distinguishable from those for the naturally occurring (−)-para-isomer of octopamine. However, at higher concentrations (above 10^{-7} mol l^{-1}) it can be seen that both NC5 and NC7 are more potent than (−)-para-octopamine and that NC5 is more potent than NC7. The effects of 10^{-7} mol l^{-1} NC5 and 10^{-7} mol l^{-1} NC7 are both blocked by 10^{-6} mol l^{-1} phentolamine, a potent blocking agent of octopamine receptors in this preparation, indicating that these effects of NC5 and NC7 are likely to be mediated *via* an action on $OCTOPAMINE_{2A}$ receptors (Evans, 1981).

The effects of NC5 and NC7 are similar on the $OCTOPAMINE_{2B}$ receptors mediating the increase in the rate of relaxation of SETi-induced twitch tension (Evans, 1981). Fig. 3 shows that both NC5 and NC7 increase the rate of relaxation of twitch tension in a dose-dependent way. As for their actions described above, both compounds are equipotent with (−)-para-octopamine, although they reach their maximal effects at 10^{-6} mol l^{-1}, an order of magnitude below that for (−)-para-octopamine. In the higher concentration range tested (10^{-6}–10^{-5} mol l^{-1}) there was a tendency for NC5 to be slightly more potent than NC7. Again the effects of 10^{-7} mol l^{-1} NC5 and 10^{-7} mol l^{-1} NC7 were blocked by 10^{-6} mol l^{-1} phentolamine,

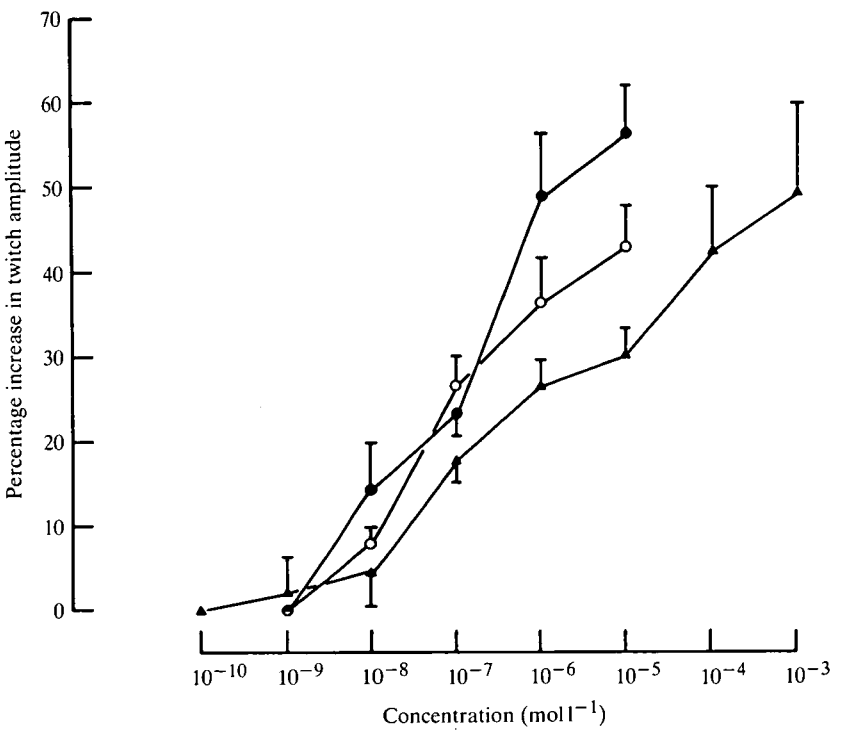

Fig. 2. Dose–response curves for the action of the phenyliminoimidazolidine derivatives NC5 (●) and NC7 (○) and (−)-para-octopamine (▲) on the maximal percentage increase of the amplitude of slow motor neurone (SETi)-induced twitch tension in the extensor-tibiae muscle of the locust hindleg. SETi was fired at a frequency of 1 Hz and each compound was introduced into the superfusate for 30 s. Each point represents the mean of at least three determinations and the bars represent standard errors.

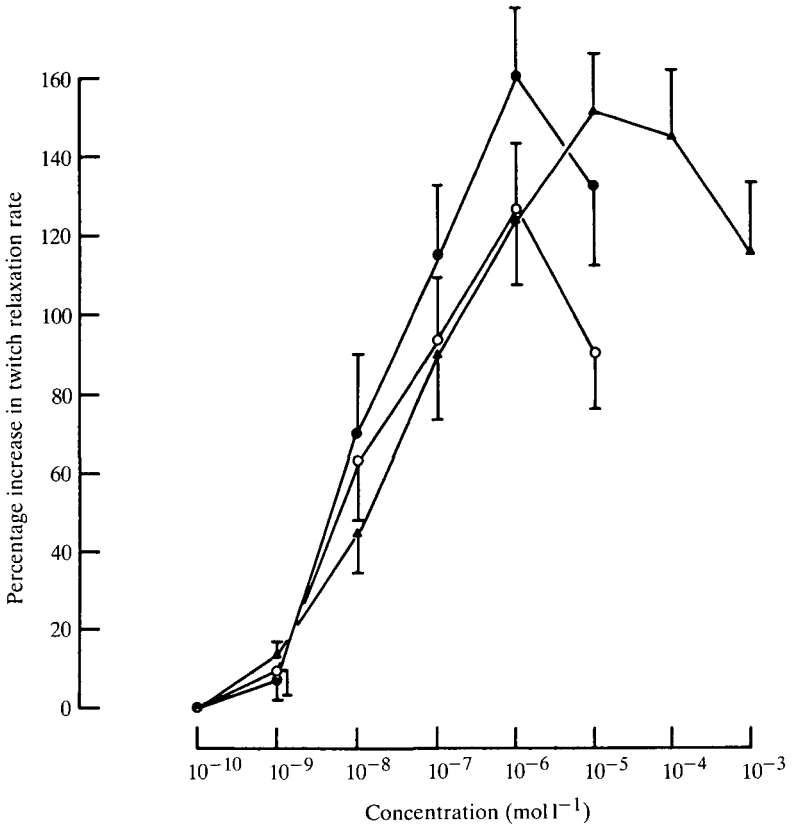

Fig. 3. Dose–response curves for the action of the phenyliminoimidazolidine derivatives NC5 (●) and NC7 (○) and (−)-para-octopamine (▲) on the maximal percentage increase in relaxation rate of slow motor neurone (SETi)-induced twitch tension in the extensor-tibiae muscle of the locust hindleg. SETi was fired at a frequency of 1 Hz and each compound was introduced into the superfusate for 30 s. Each point represents the mean of at least three determinations and the bars represent standard errors.

indicating that they are likely to be mediated *via* an action on $OCTOPAMINE_{2B}$ receptors.

Effects on $OCTOPAMINE_1$ receptors slowing myogenic rhythm

The myogenic rhythm of contraction and relaxation found in a proximal bundle of muscle fibres in the locust extensor-tibiae muscle is reduced in frequency when $OCTOPAMINE_1$ receptors are activated (Evans, 1981). Fig. 4 shows that both NC5 and NC7 are capable of mimicking this reduction in rhythm frequency in a dose-dependent way. NC7 was almost an order of magnitude more potent than (−)-para-octopamine in producing this effect and had a threshold for an observable effect of between 10^{-12} and 10^{-13} mol l^{-1}. NC5, however, was from one to two orders of magnitude less potent than (−)-para-octopamine. The effects of 10^{-9} mol l^{-1} NC7 and 10^{-7} mol l^{-1} NC5 were blocked by 10^{-6} mol l^{-1} phentolamine, indicating that

the former compounds are likely to mediate their effects by an activation of the OCTOPAMINE$_1$ subclass of octopamine receptors.

Effects on cyclic AMP levels in the extensor-tibiae muscle

OCTOPAMINE$_2$ receptors in the extensor-tibiae muscle mediate their effects *via* an activation of adenylate cyclase activity which increases the intracellular levels of the second messenger cyclic AMP (Evans, 1984*a*,*b*; 1985*b*). Both NC5 (Fig. 5) and NC7 (Fig. 6) are capable of elevating the levels of cyclic AMP in this preparation in a dose-dependent fashion. Further, the increases in levels of cyclic AMP mediated by each compound are substantially potentiated in the presence of 10^{-4} mol l^{-1} isobutylmethylxanthine (IBMX), a potent phosphodiesterase inhibitor in this preparation. However, the increases in cyclic AMP levels mediated by the phenyliminoimidazolidine derivatives are substantially less than those generated by DL-octopamine in the presence of 10^{-4} mol l^{-1} IBMX (see Figs 5, 6), despite the fact that both compounds can achieve the same maximal physiological effects as (−)-para-octopamine (see above).

A more detailed comparison of the relative increases in cyclic AMP levels produced by low concentrations of octopamine, NC5 and NC7 can be obtained by replotting the data on a log–log plot. Fig. 7 shows such a plot of the data shown in Figs 5 and 6. The octopamine-mediated increases in cyclic AMP levels are clearly

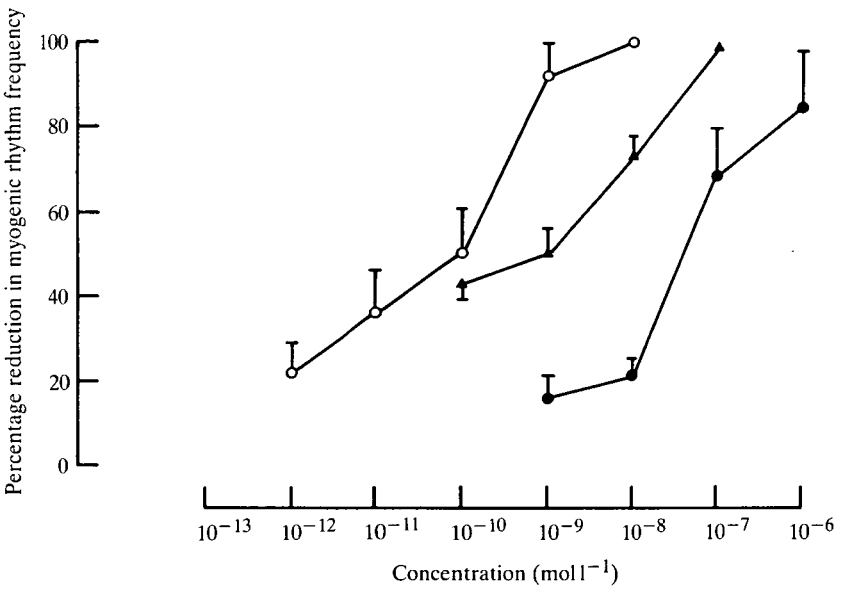

Fig. 4. Dose–response curves for the action of the phenyliminoimidazolidine derivatives NC5 (●) and NC7 (○) and (−)-para-octopamine (▲) on the slowing of the myogenic rhythm in the extensor-tibiae muscle of the locust hindleg. The results are expressed as the percentage reduction in the rhythm frequency at various concentrations of the compounds. The derivatives were introduced into the muscle superfusate for 5 min. Each point represents the mean of at least three determinations and the bars represent standard errors.

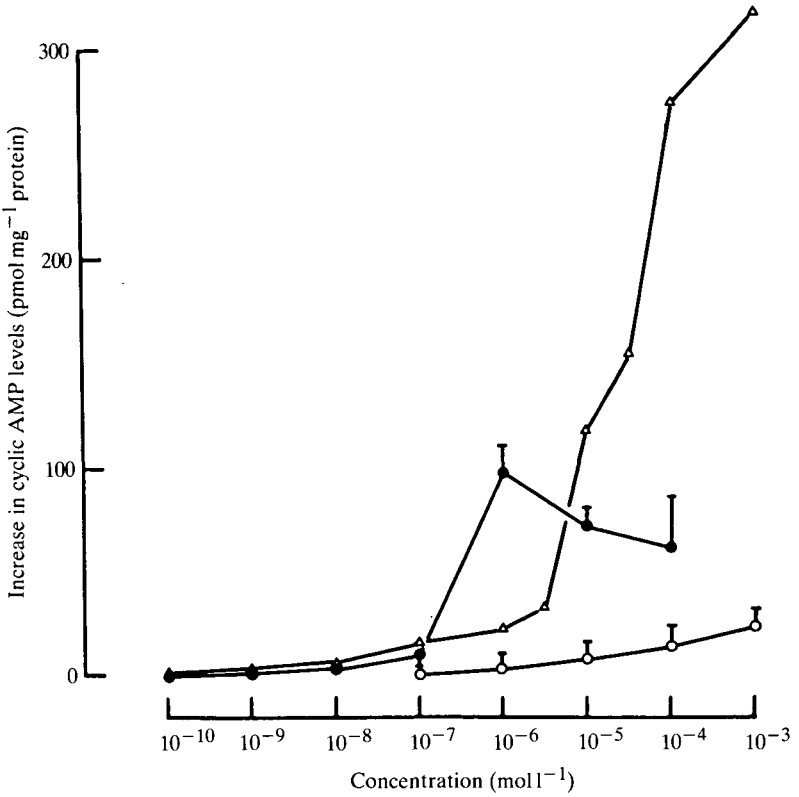

Fig. 5. Dose–response curves for the action of NC5 on the levels of cyclic AMP in the locust extensor-tibiae muscle in the presence (●) and absence (○) of 10^{-4} mol l^{-1} isobutylmethylxanthine (IBMX). The results are expressed as the increase in cyclic AMP levels (in pmol mg^{-1} protein) in the experimental muscle above that found in the contralateral control muscle. Both experimental and control muscles were pre-incubated for 10 min in 10^{-4} mol l^{-1} IBMX before exposure of the experimental muscle to NC5 plus IBMX for 10 min and the control to a further 10 min incubation in IBMX. Each value is the mean of four determinations and the bars represent standard errors. Similar data for DL-octopamine in the presence of 10^{-4} mol l^{-1} IBMX (△) are shown for comparison (taken from Evans, 1984*a*).

made up of two proportionally related components. Each component has an initial linear function slope of unity, where a ten-fold increase in octopamine concentration produces a ten-fold change in cyclic AMP accumulation. This suggests that in these regions of the curve there is no cooperativity between agonist molecules. In addition, the increases in cyclic AMP levels mediated by NC5 and NC7 overlie only the higher-affinity of the two octopamine-mediated increases in cyclic AMP level, with NC5 producing slightly bigger increases in cyclic AMP levels than NC7. The physiological dose–response curves for the actions of (−)-para-octopamine and both NC5 and NC7 on OCTOPAMINE$_2$ receptors also correspond to the higher-affinity component of the octopamine-mediated increase in cyclic AMP levels in the extensor-tibiae muscle (see Figs 2, 3).

DISCUSSION

The phenyliminoimidazolidines NC5 and NC7 can activate both OCTOPAMINE$_1$ and OCTOPAMINE$_2$ receptors on locust skeletal muscle, indicating that these compounds are not selective octopamine-2 agonists. The median effective dose (EC_{50}) for the slowing of the myogenic rhythm by NC7 (approx. 10^{-10} mol l^{-1}) (OCTOPAMINE$_1$ receptor effect) is several orders of magnitude lower than its EC_{50} for the modulation of SETi twitch tension parameters (10^{-8}–10^{-7} mol l^{-1}) (OCTOPAMINE$_2$ receptor effects). The relative potencies of NC5 and NC7, however, are different on OCTOPAMINE$_1$ and OCTOPAMINE$_2$ receptor subclasses in the locust. On OCTOPAMINE$_2$ receptors both compounds are equipotent at low concentrations, with NC5 being slightly more active than NC7 at concentrations above 10^{-7} mol l^{-1} and with both compounds being more potent than the (−)-para-isomer of octopamine. On OCTOPAMINE$_1$ receptors, NC7 is more potent than (−)-para-octopamine which, in turn, is more potent than NC5. Thus this series of phenyliminoimidazolidine derivatives, introduced by Nathanson

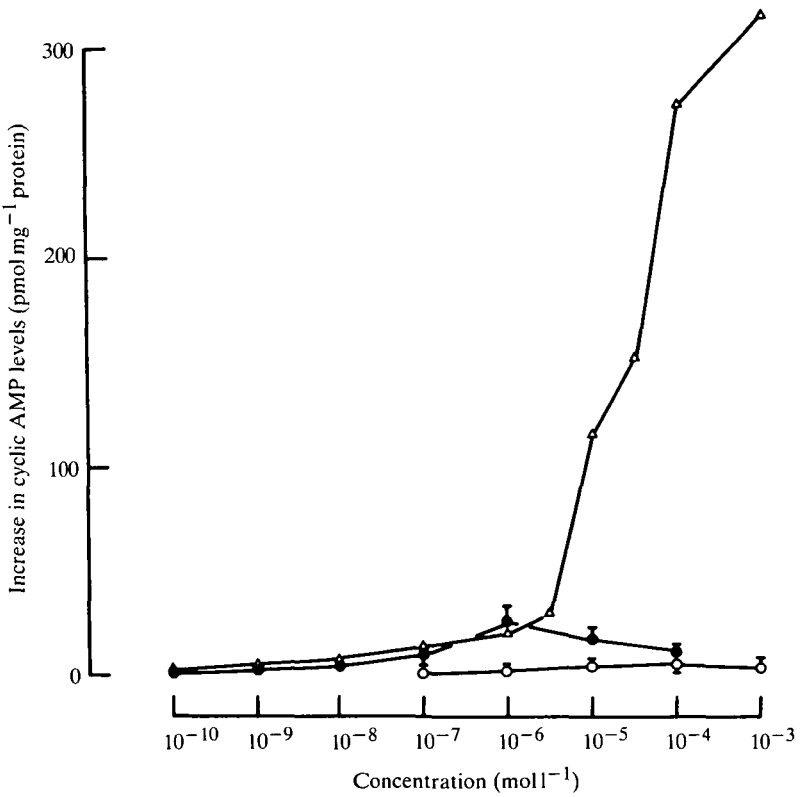

Fig. 6. Dose–response curves for the action of NC7 on the levels of cyclic AMP in the locust extensor-tibiae muscle in the presence (●) and absence (○) of 10^{-4} mol l^{-1} isobutylmethylxanthine (IBMX). Similar data for DL-octopamine in the presence of 10^{-4} mol l^{-1} IBMX (△) are shown for comparison (taken from Evans, 1984*a*). Other details as in Fig. 5.

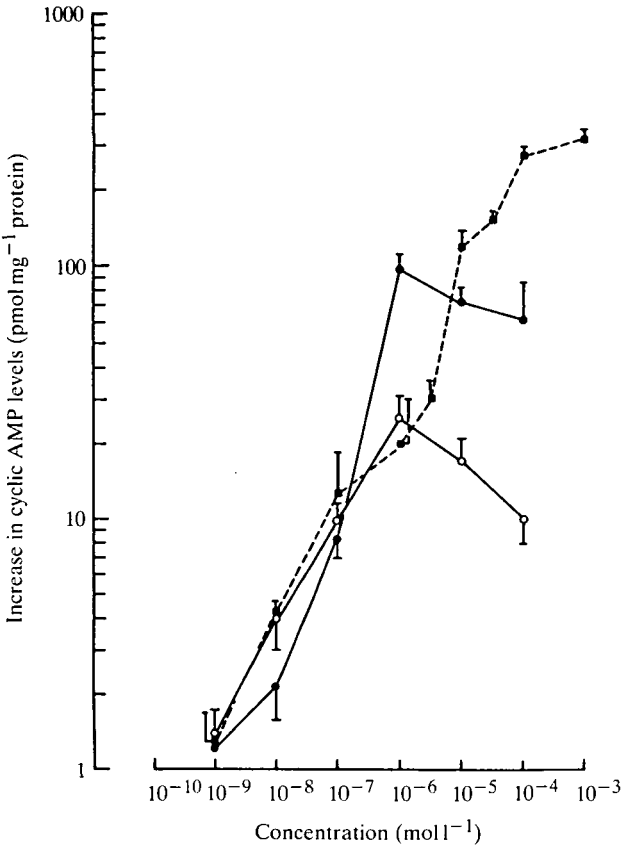

Fig. 7. A log–log plot of the actions of NC5 (●) and NC7 (○) on cyclic AMP levels in the locust extensor-tibiae muscle in the presence of 10^{-4} mol l^{-1} isobutylmethylxanthine (IBMX). The data shown are replotted from Figs 5 and 6 and the data for DL-octopamine in the presence of 10^{-4} mol l^{-1} IBMX (■) are taken from Evans & Myers (1986) for comparison. The bars represent standard errors.

(1985*a*,*b*), could well be useful in the development of selective agonists that can distinguish between the different pharmacological subclasses of octopamine receptor identified in the locust extensor-tibiae muscle preparation.

OCTOPAMINE$_2$ receptors in the locust extensor-tibiae muscle mediate their actions *via* increased cyclic AMP levels caused by an activation of the enzyme adenylate cyclase (Evans, 1984*a*,*b*). If the dose–response curve for the octopamine-mediated increase in cyclic AMP levels in the extensor muscle is plotted on a log–log plot, it can clearly be seen to be made up of two proportionally related components (Evans & Myers, 1986). At present it is not possible to identify conclusively how the two biochemically defined components relate to the OCTOPAMINE$_{2A}$ and OCTOPAMINE$_{2B}$ receptors identified pharmacologically (Evans, 1981). However, it seems very unlikely that the OCTOPAMINE$_{2A}$ sites on the presynaptic terminals of SETi could account for a substantial part of the cyclic AMP accumulation

measured in the whole muscle. Thus, it is likely that the higher-affinity component of the octopamine-mediated cyclic AMP accumulation will represent the activation of the $OCTOPAMINE_{2B}$ receptors, since the physiological responses initiated by these receptors have the same threshold and peak responses as this biochemical component. At present it has not been possible to identify any physiological response that corresponds to the lower-affinity component of the octopamine-mediated increase in cyclic AMP levels. Indeed, it may even be related to a purely biochemical effect, such as a change in carbohydrate metabolism which does not have any direct physiological equivalent.

Both NC5 and NC7 can also induce dose-dependent increases in cyclic AMP levels in the extensor muscle which are potentiated in the presence of the phosphodiesterase inhibitor IBMX. When these results are again plotted on a log–log plot they superimpose on the higher-affinity component of the octopamine curve with similar maximal effects. In contrast to the octopamine effect, they lack a second low-affinity component. This again supports the suggestion that activation of the higher-affinity component of the octopamine-mediated increase in cyclic AMP levels is sufficient to bring about the observed physiological effects on the $OCTOPAMINE_{2B}$ receptors, since both NC5 and NC7 produce maximum physiological responses that match those produced by octopamine. In addition, the plateau level of cyclic AMP accumulation produced by NC5 is slightly higher than that produced by NC7 and this closely parallels their physiological effects on the $OCTOPAMINE_2$ receptors. Thus NC5 and NC7, although not specific agonists for $OCTOPAMINE_2$ receptors, appear to be able to differentiate between the receptors mediating the two components of the octopamine-sensitive increase in cyclic AMP levels in the extensor muscle.

It has also been suggested that differences in the relative potencies of NC7 and NC5 in stimulating adenylate cyclase activity in homogenates of nervous tissue from different insect species indicate possible different $OCTOPAMINE_2$ receptor subtypes (Nathanson, 1985*a*,*b*). Thus, NC5 is more potent than NC7 in stimulating adenylate cyclase activity in homogenates of firefly light organs, whilst the converse is true in homogenates of tissue from *Manduca* central nervous system. In addition, both compounds are equipotent at stimulating this activity in homogenates of cockroach ganglia. This interpretation is complicated by the possibility of genetic differences between the $OCTOPAMINE_2$ receptors in different species and by the fact that some of the observed differences in cyclic AMP accumulation in these studies could also be due to the actions of phenyliminoimidazolidine derivatives on non-octopaminergic receptors (Nathanson, 1985*b*). However, extensive pharmacological studies (Nathanson, 1985*a*,*b*) suggest that in identified vertebrate preparations these derivatives are only weak agonists of dopamine and β-adrenergic-sensitive adenylate cyclases and that they have a low binding affinity for α_2-adrenergic receptors. These observations have led to the suggestion that the phenyliminoimidazolidines could be used for the development of pesticides with low vertebrate toxicity (Nathanson, 1985*a*,*b*). At present such a conclusion would seem

to be premature in the absence of any direct measurements of the vertebrate toxicity of these derivatives.

The finding that NC5 and NC7 are full agonists of the physiological effects of octopamine on the locust extensor muscle, whilst only activating one of the components of the octopamine-mediated increases in cyclic AMP levels in this preparation, could also explain a similar paradox observed for the actions of both formamidine pesticides, such as chlordimeform and demethylchlordimeform (Evans & Gee, 1980; Davenport, Morton & Evans, 1985), and benzylimidazolines, such as tolazoline and naphazoline (Evans, 1981, 1984*a*), on this preparation. In both cases the compounds produce similar maximal physiological responses to octopamine whilst only producing low increases in cyclic AMP levels. The latter correspond to the maximal value for the high-affinity component of the octopamine-mediated increase in cyclic AMP levels described here.

The present results support the idea that NC7 and NC5 can provide useful structures for the development of pesticides that are selective for octopamine receptors and their various subtypes. Such compounds will also be of much use as pharmacological tools for the study of the functional roles of the different octopamine receptor subtypes in the insect central nervous system.

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